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Antimicrobial susceptibility and distribution of inhibition zone diameters of bovine mastitis pathogens in Flanders, Belgium

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ABSTRACT

In dairy farms, antimicrobial drugs are frequently used for treatment of (sub)clinical mastitis. Determining the antimicrobial susceptibility of mastitis pathogens is needed to come to a correct use of antimicrobials. Strains of *Staphylococcus aureus* ($n = 768$), *Streptococcus uberis* ($n = 939$), *Streptococcus dysgalactiae* ($n = 444$), *Escherichia coli* ($n = 563$), and *Klebsiella* species ($n = 59$) originating from routine milk samples from (sub)clinical mastitis were subjected to the disk diffusion method. Disks contained representatives of frequently used antibiotics in dairy. A limited number of clinical breakpoints were available through CLSI, and showed that susceptibility of *Staph. aureus*, *E. coli*, and *Klebsiella* was moderate to high. For streptococcal species however, a large variation between the tested species and the different antimicrobials was observed. In a next step, wild type populations were described based on epidemiological cut off values (EUCAST). Because of the limited number of official cut off values, the data were observed as a mastitis subpopulation and self-generated cut off values were created and a putative wild type population was suggested.

The need for accurate clinical breakpoints for veterinary pathogens is high. Despite the lack of these breakpoints, however, a population study can be performed based on the distribution of inhibition zone diameters on the condition that a large number of strains is tested.

1. Introduction

Mastitis, in its clinical or subclinical form, remains the most costly disease on dairy farms over the world. A survey on dairy herds in Flanders, the northern part of Belgium, revealed in 2003 that 41% of the cows had a subclinical intramammary infection (Piepers et al., 2007). The incidence of clinical mastitis in Belgium is hardly studied. In the Netherlands (Barkema et al., 1998) and Canada (Riekerink et al., 2008), respectively 26 and 23 cases per 100 cows per year were registered. Along with the disease, farmers are confronted with production losses, animal discomfort, disturbance in the milking routine, and treatment costs.

In Flanders, the most frequently isolated major pathogens from subclinically infected quarters are *Streptococcus uberis* and *Staphylococcus aureus*, and *Escherichia coli*, *Strep. uberis*, *Staph. aureus*, and *Streptococcus dysgalactiae* from clinical mastitis cases (MCC, 2012). Investigating the antimicrobial resistance pattern of a bacterium is frequently performed by means of the disk diffusion method in routine veterinary labs due to the practical and economic factors. To inform the farmer and/or veterinarian on the most appropriate therapy choice, clinical breakpoints are required however often unavailable for the particular combination of the pathogen/antimicrobial per

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host species. As an alternative for the division into susceptible, intermediate susceptible, or resistant, a bacterial population can be divided into "wild type" and "non-wild type" population based on the distribution of inhibition zone diameters.

In this paper, a description of the antimicrobial resistance profile of the most prevalent pathogens from bovine mastitis is presented according to both clinical and epidemiological criteria.

2. Materials and methods

2.1. Laboratory

Analyses were performed at the laboratory of the Milk Control Centre Flanders (MCC). This organization is authorized to perform the official analysis of bulk milk samples of the Flemish dairy herds, on milk quality and composition. Besides that, MCC runs a routine lab for bacteriological culturing of milk samples.

2.2. Milk samples

Quarter milk samples presented at the lab during a one-year period (September 2012 until September 2013) were taken into account. The samples were send to the lab on voluntary basis by farmers or their veterinarian, and originated from (sub)clinical intramammary infections. If multiple samples from the same farm were submitted on the same day, this set of samples was referred to as one 'dossier'.

2.3. Bacteriological culturing

For standard culturing, the guidelines of the National Mastitis Council were followed (NMC, 1999). Briefly, a 0.01 mL loop of milk was spread on a quadrant of an aesculin blood agar plate (Oxoid, Basingstoke, UK) and incubated aerobically at 37 °C for 36 ± 12 h. Milk samples showing abnormal milk were plated also onto a McConckey agar plate (Oxoid). A quarter was considered culture-positive when growth of 1 or more colonies was detected. Phenotypic features were examined after 24 and 48 h. Growth characteristics, Gram-staining and/or presence of growth on the McConckey plate were used to distinguish Gram-negative from Gram-positive bacteria. The catalase test was used to distinguish staphylococci (positive reaction) from the *Streptococcus–Enterococcus* group (negative reaction). Within the staphylococci, the DNase activity permitted to distinguish *Staph. aureus* (positive) from other staphylococci (DNase negative or intermediately positive). Isolates from the *Streptococcus–Enterococcus* group were divided based on the aesculin reaction. Within the aesculin-negative cocci, the CAMP-test allowed to distinguish *Strep. dysgalactiae* from *Streptococcus agalactiae*. Within the aesculin-positive cocci, the growth characteristics (including color), bile aesculin agar and NaCl 6.5% were used to differentiate *Strep. uberis* from other aesculin-positive cocci (enterococci, lactococci, and aerococci). If no conclusive identification could be made, the API® strep was carried out as prescribed by the manufacturer. Within Gram-negative bacteria, appearance on McConckey agar, KOH reaction, indol production, and the triple sugar iron test were used to differentiate between *Escherichia coli*, *Klebsiella* species, and others. Other bacteria including *Corynebacterium bovis*, yeast, fungi, Prototheca species, *Bacillus* species, and *Trueperella* (*Arcanobacterium*) *pyogenes* were identified through their appearance on aesculin agar and/or morphology on Gram-staining. A milk sample was defined as contaminated if >2 different colony types were present.

2.4. Antimicrobial susceptibility testing

Not all strains of the species of interest (*Staph. aureus*, *Strep. uberis*, *Strep. dysgalactiae*, *E. coli*, and *Klebsiella*) were subjected to susceptibility testing. Of each dossier (this is the set of milk samples from one farm arriving in the lab at the same day), one strain per species of interest was selected, namely the strain isolated in the quarter with the highest somatic cell count (data on somatic cell count not presented). Selected strains were subjected to the Kirby-Bauer disk diffusion method. In short, with the InoClic® system (i2a, Perols, France) used as described by the manufacturer, a clearly separate colony of the pathogen of interest was picked and suspended in 5 mL saline solution, resulting in 0.5 McFarland. The suspension was used for flooding the Mueller Hinton agar plates (i2a), and the redundant solution was discarded. Streptococci were examined on Mueller Hinton agar plates supplemented with 5% horse blood and 20 mg/L NAD (Biomerieux, France). Antibiotic impregnated paper disks were combined in two panels, for Gram-positive and Gram-negative bacteria, respectively, and disks were applied with a dispenser. The antimicrobial agents were selected according to their occurrence in commercially available products for mastitis treatment and/or dry-cow therapy. The Gram-positive panel (for *Staph. aureus* and streptococci) consisted of oxacillin, cefoxitin, ampicillin, amoxicillin/clavulanic acid, cefquinome, tetracycline, neomycin (not for streptococci), lincomycin, erythromycin, marbofloxacin, trimethoprim/sulfamethoxazole, and rifaximin. The Gram-negative panel (for *E. coli* and *Klebsiella* species) consisted of ampicillin (not for *Klebsiella*), amoxicillin/clavulanic acid, cefquinome, tetracycline, neomycin, marbofloxacin, and trimethoprim/sulfamethoxazole. Disks were purchased from i2a, except for cefquinome and rifaximin (Mast Group, Merseyside, UK). Disks contents were indicated in Table 1. After an overnight incubation at 35 ± 2 °C, plates were read with the SIR scan Micro (i2a). The reference strains *Staph. aureus* ATCC 25923 and *E. coli* ATCC 25922 were used for quality control.

2.5. Evaluation of the inhibition zone diameters

Inhibition zone diameters were first evaluated by clinical breakpoints as provided by the Clinical and Laboratory Standards Institute (CLSI) to determine resistant strains. When veterinary breakpoints (CLSI, 2008) were not available for a certain pathogen/antimicrobial combination, human breakpoints were used (CLSI, 2012). If neither of them was available, no clinical interpretation was performed. Secondly, the distribution of the inhibition zone

Table 1
Available clinical breakpoints (and origin) and epidemiological cut off values (ECOFF, www.EUCAST.org) for the wild type (WT) population for the pathogen of interest in combination with the tested antimicrobials for a specific disk content (in μg).

AB[a]	Disk content	*Staphylococcus aureus*					*Streptococcus species*					*Enterobacteriaceae*					
		Clinical breakpoint				ECOFF	Clinical breakpoint				ECOFF	Clinical breakpoint				ECOFF *E. coli*	ECOFF *Klebsiella*
		Origin[b]	R	I	S	WT	Origin	R	I	S	WT	Origin	R	I	S	WT	WT
AMP	10	CLSI VET	28	/	29	NA	CLSI VET	18	19–26	26	NA	CLSI VET	13	14–16	17	≥14	≥~~14~~
OXA	1	CLSI VET	10	11–12	13	NA	NA	–	–	–	NA	nt[d]	–	–	–	nt	nt
CFX	30	CLSI HUM	21	/	22	≥22	NA	–	–	–	NA	nt	–	–	–	nt	nt
AMC	20/10	CLSI VET	19	/	20	NA	CLSI VET	13	14–17	18	NA	CLSI VET	13	14–17	18	≥19	≥18
CFQ	30	NA[c]	–	–	–	NA	NA	–	–	–	NA	NA	–	–	–	NA	NA
TET	30	CLSI VET	14	15–18	19	≥22	CLSI VET	18	19–22	23	NA	CLSI VET	14	15–18	19	NA	NA
NEO	30	NA	–	–	–	≥14	NA	–	–	–	NA	NA	–	–	–	≥12	≥12
LIN	15	NA	–	–	–	NA	NA	–	–	–	NA	nt	–	–	–	nt	nt
ERY	15	CLSI VET	13	14–22	23	≥21	CLSI VET	15	16–20	21	NA	nt	–	–	–	nt	nt
MAR	5	CLSI VET	14	15–19	20	NA	CLSI VET	14	15–19	20	NA	CLSI VET	14	15–19	20	NA	NA
T/S	1.25/23.75	CLSI VET	10	11–15	16	≥17	CLSI VET	15	16–18	19	NA	CLSI VET	10	11–15	16	≥16	≥19
RIF	40	NA	–	–	–	NA	NA	–	–	–	NA	nt	–	–	–	nt	nt

[a] Antimicrobials used: AMP, ampicillin; OXA, oxacillin; CFX, cefoxitin; AMC, amoxicillin/clavulanic acid; CFQ, cequinome; TET, tetracycline; NEO, neomycin; LIN, lincomycin; ERY, erythromycin; MAR, marbofloxacin; T/S, trimethoprim/sulfamethoxazole; RIF, rifaximin.
[b] Origin: CLSI VET(erinary): CLSI, 2008, M31-A3; CLSI HUM(an): CLSI, 2012, M100-S22.
[c] NA, not available.
[d] nt, not tested.

diameters was studied based on epidemiological cut off values (ECOFF) provided by EUCAST (www.EUCAST.org), to determine the 'wild type' population. When ECOFF were not available for a certain pathogen/anitmicrobial combination, a cut off value was generated. Accordingly, this self-generated cut off value (SGCOFF) lead to determination of a putative wild type population. Available clinical breakpoints and ECOFF are given in Table 1.

3. Results

3.1. Overall

In total, 27,463 samples were analyzed of which 18.9% was contaminated and 50.3% culture positive (of which 5.6% were mixed infections of two pathogens). The distribution of the pathogens is given in Table 2. Of the species of interest, 768 *Staph. aureus*, 939 *Strep. uberis*, 444 *Strep. dysgalactiae*, 563 *E. coli*, and 59 isolates of *Klebsiella* species were selected and submitted to susceptibility testing. Due to laboratory circumstances, the number of tested strains for each antimicrobial slightly differed from the total number (Table 4). Erythromycin impregnated paper disks were only added to the panel since April 2013, resulting in only 393 *Staph. aureus*, 481 *Strep. uberis*, and 231 *Strep. dysgalactiae* isolates analyzed in combination with erythromycin.

3.2. Evaluation by clinical breakpoints

The percentage resistance strains per species is given in Table 3, and indicated in Table 4. Susceptibility of *Staph. aureus* was 87.3–98.2%. Both oxacillin and cefoxitin were used for detecting methicillin-resistant *Staph. aureus*. As the cefoxitin is easier to read, this molecule is preferred (CLSI, 2008) and showed 95.6% susceptible strains in this

Table 2
Overview of pathogen distribution during the study period and pathogens subjected to susceptibility testing.

Pathogen	Isolated		Susceptibility tested	
	Nb	% of pathogens	Nb	% of isolated
Staphylococcus aureus	1914	13.1	768	40.1
Staphylococcus species	3020	20.7	–	–
Streptococcus uberis	2526	17.3	939	37.2
Streptococcus dysgalactiae	849	5.8	444	52.3
Streptococcus agalactiae	110	0.8	–	–
Other aesculin-positive cocci	606	4.2	–	–
Escherichia coli	1165	8.0	563	48.3
Klebsiella species	110	0.8	59	53.6
Other Gram-negative bacteria	231	1.6	–	–
Corynebacterium bovis	3188	21.9	–	–
Trueperella pyogenes	120	0.8	–	–
Bacillus species	184	1.3	–	–
Yeasts	505	3.5	–	–
Prototheca species	38	0.3	–	–
Fungi	7	0.0	–	–

Table 3
Evaluation of the mastitis strains by clinical breakpoints (% resistant strains) and by epidemiological cut off values (% wild type population, WT). If the latter were not available for a specific pathogen/antimicrobial combination, self-generated cut off values (SGCOFF in mm) were proposed and putative wild type population derived (% putative wild type, % pWT).

AB[a]	*Staphylococcus aureus*				*Streptococcus uberis*				*Streptococcus dysgalactiae*				*Escherichia coli*				*Klebsiella* species			
	R (%)	WT (%)	SGCOFF (mm)	pWT (%)	R (%)	WT (%)	SGCOFF (mm)	pWT (%)	R (%)	WT (%)	SGCOFF (mm)	pWT (%)	R (%)	WT (%)	SGCOFF (mm)	pWT (%)	R (%)	WT (%)	SGCOFF (mm)	pWT (%)
AMP	87.3	–	≥27	87.8	98.4	–	≥22	99.8	99.3	–	≥24	99.8	71.2	87.6	–	–	nt	nt	nt	nt
OXA	97.4	–	≥18	95.7	–	–	≥21	31.8	–	–	≥20	99.1	nt	nt	nt	nt	nt	nt	nt	nt
CFX	95.6	95.6	–	–	–	–	≥19	98.8	–	–	≥22	99.8	nt	nt	nt	nt	nt	nt	nt	nt
AMC	97.9	–	≥28	89.3	99.9	–	≥29	96.9	99.8	–	≥30	96.6	92.7	90.1	–	–	98.3	98.3	–	–
CFQ	–	–	≥25	97.4	–	–	≥25	99.1	–	–	≥30	97.2	–	–	≥25	97.1	–	–	≥25	96.6
TET	91.8	91.7	–	–	59.2	–	≥22	59.2	6.8	–	≥13	56.0	85.3	–	≥17	86.9	83.1	–	≥17	88.1
NEO	–	99.6		–	nt[b]	nt	nt	nt	nt	nt	nt	nt	–	94.8	–	–	–	98.3	–	–
LIN	–	–	≥23	87.4	–	–	≥17	42.4	–	–	≥17	67.5	nt	nt	nt	nt	nt	nt	nt	nt
ERY	96.9	98.5	–	–	69.6	–	≥18	77.1	80.5	–	≥18	87.0	nt	nt	nt	nt	nt	nt	nt	nt
MAR	96.6	–	≥21	96.5	80.0	–	≥16	98.6	70.3	–	≥16	99.1	96.6	–	≥26	94.7	96.6	–	≥26	96.6
T/S	98.2	97.9	–	–	97.5	–	≥17	98.4	98.6	–	≥16	99.1	90.4	90.4	–	–	96.6	96.6	–	–
RIF	–	–	≥21	99.9	–	–	≥20	99.2	–	–	≥20	100.0	nt	nt	nt	nt	nt	nt	nt	nt

[a] Antimicrobials used: AMP, ampicillin; OXA, oxacillin; CFX, cefoxitin; AMC, amoxicillin/clavulanic acid; CFQ, cequinome; TET, tetracycline; NEO, neomycin; LIN, lincomycin; ERY, erythromycin; MAR, marbofloxacin; T/S, trimethoprim/sulfamethoxazole; RIF, rifaximin.
[b] nt, not tested.

study (compared to 97.4% susceptible for oxacillin). No further investigation was performed to confirm methicillin resistance. The number of susceptible *Streptococcus* strains varied largely between the two species tested and between the different antimicrobials, namely 59.2–99% for *Strep. uberis* versus 6.8–99.3% for *Strep. dysgalactiae*. *E. coli* strains were moderate to highly susceptible (71.2–96.6%), and *Klebsiella* had rather high susceptibility rates (83.1–98.3%).

3.3. Evaluation by epidemiological cut off values

The distribution of the mastitis strains over the inhibition zone diameters is presented (Table 4) and the percentage of strains belonging to the wild type population was calculated (Tables 3 and 4). Wild type population of *Staph. aureus* (5 ECOFF available out of 12 pathogen/antibiotic combinations) enclosed between 91.7% (tetracycline) and 99.6% (neomycin) of the tested strains. No ECOFF for *Strep. uberis* nor *Strep. dysgalactiae* for the given antibiotics were available through EUCAST. Wild type population of *E. coli* (4/7 ECOFF available) included between 87.6% (ampicillin) and 94.8% (neomycin) of the tested strains, and of *Klebsiella* species (3/6 ECOFF available) between 96.6% (trimethoprim/sulfamethoxazole) and 98.3% (amoxicillin/clavulanic acid and neomycin).

Self-generated cut off values (SGCOFF) as derived from the distribution of inhibition zone diameters (Table 4) and putative wild type populations are given in Table 3. For most pathogen/antimicrobial combinations, a clear separation between the two peaks of the bimodal curve was observed. The putative wild type population of *Staph. aureus* enclosed 87.4–99.9% of the strains, of *Strep. uberis* 31.8–99.8%, of *Strep. dysgalactiae* 56.0–100%, of *E. coli* 86.9–97.1%, and of *Klebsiella* 88.1–96.6% of the strains. The species specific SGCOFF for the streptococci were rather similar, except for tetracycline (22 versus 13 mm, for *Strep. uberis* and *Strep. dysgalactiae*, respectively).

4. Discussion

In this paper, antimicrobial susceptibility of frequently isolated major pathogens (*Staph. aureus*, *Strep. uberis* and *Strep. dysgalactiae*, *E. coli*, and *Klebsiella* species) originating from milk samples of (sub)clinical mastitis in dairy herds in Flanders was presented. The samples were not originating from a random sample of herds and cows but were recovered during a one-year period in the routine lab. The followed protocol prescribed to select only one isolate of each pathogen of interest from each dossier for susceptibility testing, due to economic restrictions. The farmers are strongly advised to take current and historical data into account to set up a treatment protocol, in order to overcome the limited number of susceptibility tests per dossier. This data set presented is thus a convenience sample, however, as a large number of strains is included from different herd types it will give an acceptable insight in the Flemish situation. Growth of a single colony from a 0.01 mL milk sample, on a single sampling occasion, was defined as a positive sample. This protocol provides high sensitivity but lower specificity compared to a proposed

Table 4
Distribution of inhibition zone diameters in mm for each pathogen and antimicrobial tested, and number of tested strains per combination. Clinical breakpoints (if available through CLSI) are indicated with a vertical dash, wild type population based on EUCAST epidemiological cut off values is indicated in dark gray. Putative wild type population based on self-generated cut off values (determined when no EUCAST cut off values were available for specific pathogen/antimicrobial combinations) is indicated in light gray.

	N	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
Staphylococcus aureus																																				
AMP	761	0.0	0.1	0.0	0.3	1.1	0.1	1.1	0.9	1.3	0.9	0.8	0.3	0.8	0.0	0.8	1.2	1.1	0.5	0.4	0.4	0.3	0.1	0.4	0.3	0.3	0.1	0.5	0.5	0.5	0.3	0.5	0.4	2.2	4.2	77.4
OXA	768	1.6	0.1	0.0	0.4	0.0	0.1	0.4	0.1	0.4	0.5	0.4	0.3	0.3	0.4	2.1	2.9	3.1	4.0	5.2	4.2	5.2	4.4	5.5	6.3	4.8	4.7	4.9	4.4	5.1	5.5	2.9	2.9	2.0	0.1	15.0
CFX	768	0.8	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.7	0.7	0.3	0.8	0.3	0.3	0.1	0.1	0.0	0.3	0.1	1.2	1.0	8.6	7.6	6.9	7.3	9.8	7.6	9.5	8.1	5.7	5.6	3.1	2.9	0.5	9.9
AMC	755	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.1	0.3	0.1	0.4	0.3	0.3	0.5	0.8	0.7	1.2	1.7	0.9	1.2	0.9	1.2	0.4	0.5	0.7	0.5	0.5	0.5	0.4	0.3	0.3	0.1	0.4	1.3	83.3
CFQ	760	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.7	0.9	0.8	0.3	0.7	0.4	1.8	2.4	3.6	6.6	9.2	11.2	11.8	12.8	10.3	10.7	8.9	3.8	3.0
TET	768	0.7	0.5	1.0	0.7	1.3	0.9	1.3	0.8	0.5	0.3	0.1	0.0	0.1	0.1	0.0	0.0	0.3	0.4	0.7	1.0	1.8	3.6	3.5	8.9	15.2	15.0	16.0	12.4	6.8	3.3	1.8	0.3	0.3	0.0	0.5
NEO	768	0.0	0.0	0.0	0.1	0.0	0.0	0.1	0.1	0.1	0.0	0.0	1.3	4.7	14.2	24.1	28.8	13.9	4.9	3.1	1.2	1.3	0.3	0.3	0.1	0.0	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0
LIN	767	3.3	0.0	0.3	0.9	0.9	0.9	0.7	0.7	0.4	0.8	0.5	0.7	0.7	0.7	0.4	0.8	0.3	0.1	0.4	1.2	0.7	2.3	3.7	5.1	7.6	11.1	12.9	13.8	13.7	7.7	3.4	1.6	1.3	0.1	0.8
ERY	393	0.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.3	0.0	0.0	0.3	0.3	1.3	2.8	5.3	10.2	18.6	20.4	15.3	10.7	6.6	1.8	1.5	0.0	0.3	0.0	0.0	0.0	0.0	0.0	3.6
MAR	767	1.8	0.0	0.3	0.5	0.0	0.0	0.0	0.1	0.0	0.0	0.1	0.3	0.1	0.1	0.1	0.3	0.4	1.4	3.5	12.4	19.6	18.1	14.9	13.2	6.8	2.9	1.4	0.3	0.1	0.4	0.1	0.0	0.1	0.0	0.7
T/S	768	1.3	0.0	0.1	0.0	0.0	0.1	0.0	0.1	0.1	0.0	0.3	0.4	0.3	0.5	0.7	0.9	0.4	1.2	0.4	0.9	1.0	5.1	9.9	19.1	21.4	16.4	10.4	5.6	1.8	0.8	0.1	0.0	0.0	0.0	0.7
RIF	765	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.7	0.1	1.0	2.6	2.0	3.3	4.2	5.5	4.6	5.2	5.6	11.0	13.3	9.2	7.3	4.4	1.7	0.1	17.9
Streptococcus uberis																																				
AMP	937	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.2	0.2	0.4	0.5	0.4	0.9	2.9	4.2	6.8	9.2	9.9	12.2	11.5	10.1	11.0	7.2	2.8	1.0	8.4
OXA	938	2.1	0.1	0.3	1.0	1.6	3.0	5.3	4.8	7.9	8.3	9.5	8.4	6.9	5.9	3.1	1.9	3.2	2.9	4.5	4.5	3.3	2.7	2.3	1.2	1.2	0.6	0.4	0.5	0.2	0.0	0.0	0.0	0.0	0.0	2.3
CFX	937	0.4	0.1	0.0	0.0	0.1	0.1	0.0	0.0	0.3	0.0	0.0	0.0	0.1	0.0	0.2	0.1	2.8	4.3	6.6	8.3	10.2	14.7	11.2	9.7	7.5	5.5	4.5	4.4	2.9	1.5	0.5	0.4	0.2	0.0	3.2
AMC	939	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.6	0.4	0.3	0.1	0.9	0.5	0.4	1.1	2.3	2.8	4.9	6.5	8.7	11.2	12.0	11.7	3.0	32.3
CFQ	932	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.5	0.2	0.4	0.3	1.1	2.0	2.4	4.6	4.4	7.5	10.2	11.3	12.0	8.3	6.7	3.9	3.3	20.8
TET	939	13.4	0.6	3.5	4.0	4.0	3.9	3.7	2.6	1.3	1.4	0.4	0.2	0.6	0.6	0.2	0.1	0.0	1.4	1.8	5.2	5.9	6.9	7.7	9.2	5.5	4.9	3.8	1.3	1.4	1.0	1.0	1.4	0.6	0.1	0.2
LIN	937	48.2	0.0	1.6	1.3	1.3	0.9	1.1	0.7	1.3	0.4	0.9	0.1	1.0	1.7	2.7	5.8	3.3	4.2	2.9	1.8	2.6	2.0	2.1	1.9	3.2	2.1	1.2	1.2	0.7	0.3	0.5	0.1	0.0	0.0	1.0
ERY	481	10.6	0.0	0.2	0.6	1.2	2.5	1.5	1.5	0.6	1.0	1.5	1.7	1.7	3.1	2.7	3.7	2.5	3.7	6.2	6.2	8.5	8.7	7.5	4.2	4.4	2.3	2.1	1.7	0.6	1.0	0.6	0.4	0.6	0.0	4.6
MAR	936	1.1	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.1	0.1	0.0	0.9	5.8	12.0	16.8	18.4	15.2	10.4	6.4	4.7	2.8	1.6	0.9	0.7	0.4	0.3	0.6	0.1	0.3	0.2	0.0	0.0	0.0	0.0	0.2
T/S	937	1.2	0.0	0.1	0.0	0.1	0.1	0.0	0.1	0.0	0.0	0.0	0.3	0.5	1.6	2.8	6.1	9.7	12.6	13.9	13.1	11.6	7.8	4.6	3.4	1.9	1.9	1.1	1.7	1.1	1.0	0.6	0.0	0.4	0.1	0.5
RIF	933	0.0	0.0	0.0	0.2	0.0	0.0	0.1	0.3	0.0	0.0	0.0	0.1	0.0	0.0	0.2	0.3	1.3	2.7	4.9	9.6	14.1	15.0	13.8	10.9	7.9	5.9	3.6	2.7	1.5	0.6	0.6	0.2	0.3	0.1	2.7
Streptococcus dysgalactiae																																				
AMP	443	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.2	0.2	0.5	0.2	0.7	0.5	1.8	2.9	3.6	7.7	14.0	17.8	15.1	12.0	8.8	1.6	12.2
OXA	442	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.2	0.0	0.2	0.2	0.0	0.5	1.1	0.7	1.6	4.8	8.4	15.6	14.7	16.3	11.3	6.8	2.7	2.5	1.6	1.1	0.5	0.2	0.2	0.0	0.2	8.4
CFX	444	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.5	0.5	1.1	2.0	5.2	9.0	18.5	14.2	13.5	9.0	8.1	5.4	2.9	1.6	0.7	0.2	0.0	7.0
AMC	442	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.2	0.5	0.5	0.2	1.4	0.5	0.5	1.8	1.6	2.9	5.7	9.0	13.6	14.7	2.7	43.7
CFQ	434	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.2	0.2	1.2	0.9	0.5	0.9	2.3	5.1	7.4	13.8	11.5	13.4	5.8	5.8	30.9
TET	443	5.6	0.0	7.7	9.7	9.9	7.0	4.1	1.4	2.3	2.3	3.6	5.0	8.8	7.2	7.2	6.1	5.4	2.0	1.1	0.9	0.9	0.5	0.2	0.0	0.2	0.2	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.5
LIN	443	26.9	0.2	0.5	0.5	0.0	0.9	0.5	0.7	0.7	0.7	1.1	0.5	1.1	0.9	2.7	1.8	1.6	1.8	1.8	3.4	7.7	8.1	7.7	7.2	4.5	5.9	2.7	1.4	1.6	1.1	0.5	0.5	0.0	0.0	3.2
ERY	231	7.8	0.0	0.0	0.0	0.9	0.9	0.9	0.4	0.0	0.9	0.4	0.9	1.7	0.4	4.3	2.6	5.6	8.7	10.0	7.4	13.9	10.8	6.1	1.3	1.7	1.3	0.4	1.7	1.3	1.3	0.0	0.4	0.0	0.4	5.6
MAR	444	0.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.7	9.9	17.8	22.3	18.0	9.0	6.1	4.1	2.7	1.8	0.9	1.1	1.1	0.5	0.5	0.5	0.5	0.2	0.0	0.0	0.0	0.0	0.0	1.1
T/S	440	0.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.5	0.9	0.7	0.2	3.6	6.1	8.2	13.0	17.3	13.4	11.1	7.7	4.8	3.2	1.6	1.4	0.9	1.4	0.7	0.5	0.0	1.6
RIF	442	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	2.0	3.6	12.0	14.5	19.9	14.7	11.5	7.2	2.7	1.8	2.3	1.4	1.1	0.0	0.0	0.0	0.0	5.0

Table 4
Distribution of inhibition zone diameters in mm for each pathogen and antimicrobial tested, and number of tested strains per combination. Clinical breakpoints (if available through CLSI) are indicated with a vertical dash, wild type population based on EUCAST epidemiological cut off values is indicated in dark gray. Putative wild type population based on self-generated cut off values (determined when no EUCAST cut off values were available for specific pathogen/antimicrobial combinations) is indicated in light gray.

Escherichia coli																																							
AMP	563	10.5	0.0	0.2	0.2	0.0	0.0	0.5	1.1	2.5	6.9	6.9	8.9	9.6	16.0	13.1	10.7	6.9	2.7	2.1	0.7	0.4	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
AMC	563	0.4	0.0	0.4	0.2	0.5	1.2	0.0	0.9	0.7	1.2	0.4	1.4	2.7	7.5	8.7	8.9	16.2	15.3	12.3	11.7	3.7	4.1	1.1	0.2	0.2	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
CFQ	561	0.0	0.0	0.0	0.0	0.2	0.4	0.2	0.2	0.2	0.0	0.4	0.0	0.0	0.4	0.0	0.2	0.2	0.5	0.2	0.5	0.5	2.3	3.9	7.3	16.4	16.9	18.2	16.2	8.2	2.5	1.8	1.2	0.4	0.2	0.5		
TET	563	9.2	1.2	0.7	0.2	0.4	0.2	0.2	0.0	0.2	0.2	0.7	0.5	1.1	3.0	12.4	21.1	19.4	15.5	8.2	1.8	1.8	0.4	1.1	0.2	0.2	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		
NEO	563	2.5	0.0	0.9	1.2	0.4	0.2	0.0	0.0	0.0	0.2	0.2	1.2	3.7	24.0	30.9	21.7	9.4	1.4	1.2	0.5	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2
MAR	562	1.2	0.0	0.4	0.2	0.7	0.7	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.9	0.4	0.2	0.4	0.2	0.4	0.7	4.8	6.8	10.7	17.6	17.1	14.2	10.0	7.3	2.3	2.0	0.2	0.5		
T/S	563	8.5	0.0	0.0	0.0	0.0	0.0	0.4	0.5	0.0	0.2	0.0	0.2	0.0	0.2	0.5	0.7	0.9	0.5	2.5	7.3	12.6	14.9	16.2	16.7	8.2	4.6	2.0	0.9	0.4	0.2	0.4	0.0	0.0	0.0	0.7		
Klebsiella species																																						
AMC	59	1.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.7	1.7	1.7	8.5	11.9	27.1	13.6	8.5	16.9	6.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		
CFQ	59	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.7	1.7	8.5	15.3	15.3	11.9	16.9	13.6	5.1	3.4	1.7	1.7	0.0	0.0	0.0		
TET	59	8.5	0.0	0.0	0.0	0.0	0.0	3.4	0.0	0.0	0.0	0.0	1.7	3.4	8.5	10.2	15.3	20.3	18.6	6.8	0.0	3.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		
NEO	59	0.0	0.0	0.0	0.0	0.0	1.7	0.0	0.0	0.0	0.0	1.7	1.7	6.8	15.3	28.8	22.0	10.2	6.8	5.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		
MAR	59	3.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.4	6.8	23.7	20.3	11.9	5.1	6.8	3.4	11.9	1.7	1.7	0.0	0.0	0.0	0.0		
T/S	59	1.7	0.0	0.0	0.0	0.0	1.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.7	5.1	3.4	13.6	18.6	11.9	18.6	6.8	6.8	3.4	3.4	1.7	1.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		

gold standard of triplicate milk samples (Dohoo et al., 2011), which meets the objectives of most routine labs.

To examine the antimicrobial resistance of bacteria, a variety of methods is available. Routine labs strive for a quick test result and, more than in human medicine, veterinary labs are impeded by economic restrictions. Therefore, disk diffusion is most frequently used in these labs (CLSI, 2008), as is done in the lab described in this paper. For the disk diffusion method as well as other tests, however, appropriate breakpoints are a prerequisite to differentiate susceptible from resistant bacteria and to guide a therapeutic approach (clinical breakpoints). Of only 58% of the pathogen/antimicrobial combinations used in this study, the veterinary breakpoints were described in the CLSI guidelines (Table 1). Accordingly, as different research groups use different breakpoints, data on susceptibility are hardly comparable. Compared to data from Poland (Malinowski et al., 2002) and Brazil (Costa et al., 2000), both based on human CLSI breakpoints, the susceptibility of *Staph. aureus* for most antimicrobials seems acceptable in Flanders. Resistance of the two *Streptococcus* species was high, and additionally, a discrepancy within the genus was notable. For tetracycline for example, only 6.8% of the *Strep. dysgalactiae* strains was susceptible compared to 59.2% of the *Strep. uberis* strains (Table 3). Erskine et al. (2002) reported similar percentages of susceptibility of *Strep. uberis*, but a varying percentage of *Strep. dysgalactiae* was observed over the years. This finding confirms earlier data from France based on MIC testing (Guérin-Faublée et al., 2002), although others reported low tetracycline activity (MIC) for both species (Rossitto et al., 2002). For *E. coli*, susceptibility was comparable to previous studies based on disk diffusion methods as for trimethoprim/sulfamethoxazole (Erskine et al., 2002). Tetracycline activity was moderate (85.3% susceptible) but higher than reported elsewhere (Erskine et al., 2002). Studies based on MIC described a higher activity of ampicillin and tetracycline (Guérin-Faublée et al., 2003; Bengtsson et al., 2009). Erskine et al. (2002) found somewhat higher susceptibility for ampicillin (85.5%). The decreased susceptibility of *E. coli* to ampicillin (71.2%) was not further studied. A partial explanation might be found in the exposure (as being part of the gastrointestinal microbiota) to this and analogous molecules. Using the available CLSI breakpoints, *Klebsiella* species showed high susceptibility (Table 3) and in particular higher susceptibility for tetracycline compared to other disk diffusion based reports (Erskine et al., 2002).

Next to the evaluation based on clinical breakpoints, an epidemiological evaluation was performed. Again, the unavailability of appropriate cut off values was encountered. Of only 26% of the pathogen/antimicrobial combinations used in this study, the ECOFF were described in the EUCAST guidelines (Table 1). No ECOFF were available for the two tested *Streptococcus* species. Since a considerable number of strains were tested in this study, the presented data can be regarded as a mastitis-subpopulation enhancing the creation of (self-generated) cut off values based on the histograms of the inhibition zone diameters (histograms not shown; Table 4). This way, a 'putative wild type' population was derived whenever ECOFF were absent

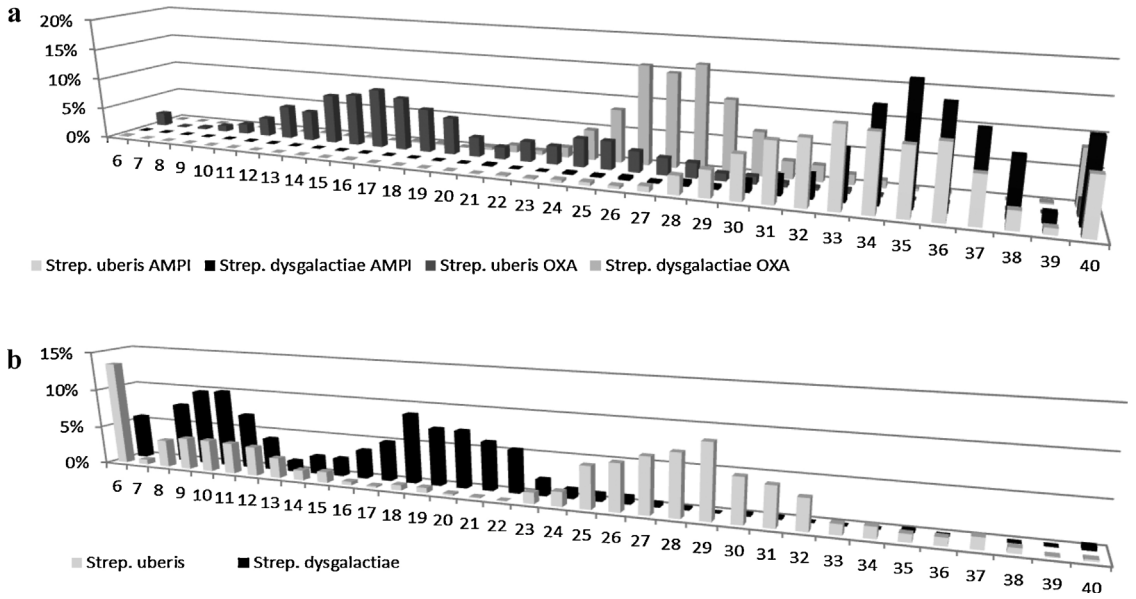

Fig. 1. Distribution of inhibition zone diameters of *Streptococcus uberis* and *Strep. dysgalactiae* strains isolated from bovine mastitis for (a) ampicillin and oxacillin, and (b) tetracycline, showing a comparable distribution for ampicillin but different distributions for oxacillin and tetracycline within the genus *Streptococcus*.

(Table 3). For most pathogen/antimicrobial combinations a bimodal distribution was observed indicating acquired resistance in some strains (Table 4). For *Staph. aureus* tested against ampicillin and amoxycillin/clavulanic acid, this separation of two populations based on the curve was less obvious. The SGCOFF was set at 27 resp. 28 mm but need to be further studied with more strains. For *Strep. uberis* against oxacillin a bimodal curve was also displayed, but on the contrary, the majority of strains was situated at smaller zone diameters (putative non-wild-type) (Fig. 1a). This division of the population into wild and non-wild-type was ambiguous (Table 4). Only 31.8% of the strains were putatively assigned to the wild type population (SGCOFF at $\geq$21 mm, Table 3). In Belgium, cloxacillin is frequently used for (intramammary) mastitis treatment, and its equivalent oxacillin is added to the antimicrobial panel upon request of the veterinarians. The use of oxacillin disks to examine streptococci could be discussed, as no clinical breakpoints nor epidemiological cut off values are available and, in addition, penicillin is the preferred molecule to test beta-lactam susceptibility. It seemed however that *Strep. dysgalactiae* followed a different distribution, with 99.1% of the strains belonging to the putative wild type population (SGCOFF at $\geq$20 mm) indicating that testing oxacillin might be possible for streptococci. For other antimicrobials of the beta-lactam group (e.g. ampicillin), this difference between the streptococcal species is not visible (Fig. 1a). Also for tetracycline, a species specific distribution of the inhibition zone diameter is shown within the tested streptococci. *Strep. uberis* displayed a histogram with two clearly distinguished peaks and the SGCOFF was set at $\geq$22 mm. Still, only 59.2% of the strains were categorized as putative wild type population. For *Strep. dysgalactiae*, a comparable percentage of strains was defined as putative wild type (56.0%), however, this SGCOFF was set at a much lower inhibition zone diameter ($\geq$13 mm). The histogram was clearly shifted to the left compared to *Strep. uberis* (Fig. 1b). Whether or not these interspecies incongruence is merely a laboratory observation or this has also an effect in the field, has to be determined. Obviously, veterinary cut off values (as well as clinical breakpoints) for mastitis pathogens should be defined with detail on species level, at least for *Streptococcus* species as it is a very heterogeneous group of organisms.

5. Conclusion

Clinical breakpoints as well as epidemiological cut off values are often unavailable for studying veterinary pathogens by disk diffusion or other methods, and in addition, epidemiological cut off values and clinical breakpoints are not (necessarily) linked (Schwarz, 2010). Analysing data based on epidemiological criteria is not the main objective of many routine labs. However, these routine labs often have access to a large amount of data which can be used to perform studies on specific populations. By using a high number of strains, a glance at the situation in the field of bovine mastitis in Flanders is provided in this study.

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